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Sheffield medium for cultivation of *Haemophilus ducreyi*

S HAFIZ,* M G McENTEGART,* AND G R KINGHORN†

*From the *Department of Medical Microbiology, University of Sheffield Medical School, and the †Department of Genitourinary Medicine, Royal Hallamshire Hospital, Sheffield*

SUMMARY Our interest in the role of *Haemophilus ducreyi* in genital ulceration led us to examine the various media commonly used for the cultivation of the organism. We describe an improved medium for the routine isolation of *H ducreyi*. In comparative studies using 50 test strains originally isolated in the United Kingdom, Canada, the United States of America, and Kenya, the new medium proved superior to three standard media in requiring a shorter incubation period to first visible growth, giving larger colonies in the same period, and making possible a starch aggregation test which we have found helpful in the presumptive identification of *H ducreyi* from clinical material.

Introduction

Haemophilus ducreyi is a fastidious organism which was, until recently, considered to be so difficult to grow that the diagnosis of chancroid was commonly based on the microscopical appearance of stained films of ulcer exudate and on the exclusion of all other causes of genital ulceration.[1] The introduction of improved culture methods for its isolation[2-5] has helped to make the laboratory identification of *H ducreyi* reliable, and the diagnosis of chancroid therefore more certain. Nevertheless the media currently used may still pose problems, especially in needing a long incubation time, giving variable colony sizes, and showing diverse microscopical appearances on Gram stained smears prepared from colonies. In this study we compared an improved Sheffield medium with other standard media in cultivating strains of *H ducreyi* originally isolated from patients attending sexually transmitted disease (STD) clinics.

Materials and methods

STRAINS OF *H DUCREYI*

We studied 50 strains of *H ducreyi*, 25 from patients attending the department of genitourinary medicine at this hospital, 15 from patients with chancroid in Canada and Kenya (supplied by Dr A Ronald), four from patients with chancroid in Seattle (supplied by Dr H Handsfield), five isolated in Orange County, California (supplied by Dr J R Greenwood), and the NCTC (National Collection of Type Cultures) strain 10945.

Address for reprints: Dr S Hafiz, Department of Medical Microbiology, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX

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MEDIA

The four media used were: (a) enriched chocolate agar[2] consisting of GC base (Difco) and distilled water with 1% haemoglobin and 1% isovitalex added as supplements; (b) fetal bovine serum agar[3] consisting of 40 g of heart infusion agar (Difco) dissolved in 900 ml distilled water and sterilised by autoclaving, to which 100 ml of fetal bovine serum were added as supplement; (c) modified haemin medium[5] consisting of GC base (Difco), proteose peptone, gelatin, defined supplement, and 250 mg/l of equine haemin type III (Sigma); and (d) Sheffield medium, which was an improvement of the modified haemin medium and was prepared as follows:

Part I

Thirty g proteose peptone no 3 (Difco), 1 g rice starch, 4 g potassium phosphate (dibasic), 1 g potassium phosphate (monobasic), 5 g sodium chloride, 4 g gelatin, and 10 g agar were soaked in 1000 ml distilled water for 30 minutes, mixed, sterilised by autoclaving at 67 kPa (10 lbs/in^2) pressure and 115°C for 15 minutes, and cooled to 56°C in a water bath.

Part II (Stable defined supplement)
One g gelatin and 1 g glutamine were dissolved in 90 ml distilled water at 100°C, 40 g glucose was added, and the gelatin solution was cooled to about 10°C. A solution of 0·05 g ferric nitrate was dissolved without heating in 10 ml distilled water, mixed with the gelatin solution by stirring vigorously, distributed in 10 ml amounts, sterilised by autoclaving at 67 kPa (10 lbs/in^2 pressure and 115°C for 15 minutes, and stored in the dark until required.

Part III
One g of equine haemin type III (Sigma) was dissolved in 25 ml of 0·2 ml potassium hydroxide in 47·5% ethanol, and 75 ml distilled water was added. The solution was sterilised by filtration and distributed in 20 ml amounts.

Sheffield medium
The final medium was made by mixing one litre Part I, 10 ml Part II, and 20 ml Part III and adding 5% horse defibrinated blood (Oxoid Code SR50) to give better growth. The pH of the medium should be 7·0-7·2, and if it was lower than 7·0 it was adjusted by adding N/10 (one tenth normal) sodium hydroxide.

INOCULA
All identified strains of *H ducreyi* were cultured on Hammond's medium[2] and on modified haemin[5] medium and then incubated at 33-34°C in 5% carbon dioxide for 96 hours. Growth from each of these cultures was scraped off, suspended in phosphate buffered saline (PBS), mixed on a Vortex mixer for five minutes, and left for 10 minutes for the larger lumps to settle. A standard loopful of each of these suspensions was inoculated on to seven replicates of the four media tested. Inoculated plates were incubated under the optimum conditions described below and results were read every 24 hours for seven days, the plates being examined for typical type, size, coherence, and starch aggregation.[6] Films were also prepared by Gram's staining and examined for typical morphology. Preliminary experiments using NCTC strain 10945 and several local isolates on all four media had shown that inoculated plates incubated at 37°C gave less growth than at 33-34°C, or none.

Results

On all four media, the strains produced characteristic *H ducreyi* colonies which were difficult to emulsify and could be pushed intact across the surface of the plate.

The table shows that colonies were largest on the new medium, which also gave visible growth after incubation for 48 hours with 40 of the 50 strains tested, and with all 50 strains after incubation for four days. In this respect the new medium was superior to the other three media tested. Gram stained smears of colonies from the new medium more often showed the typical "rail road tracks" appearance, as shown in the figure.

Starch aggregation[6] was seen on modified haemin medium after four days, but after only two days with the new medium. It was not seen on either Hammond's (enriched chocolate agar)[2] or Sottnek's (fetal bovine serum agar)[3] medium.

Discussion

With the introduction of reliable culture methods our concept of *H ducreyi* has changed from that of a rare organism which was extremely difficult to isolate to an organism which, with care, can be grown and identified in a routine laboratory. We found that the

TABLE *A comparative growth of 50 strains of H ducreyi inoculated on four media*

Medium	*No of strains showing visible growth on days:* 1	2	3	4	5	6	7	*Size and colour of colonies*	*Appearance on Gram's staining*	*Starch aggregation*
Enriched chocolate agar		25	35	45	50	50	50	Small non-mucoid yellow-grey translucent	Gram negative rods in clumps or short chains on occasional rail road tracks	Negative
Fetal bovine serum agar		22	31	42	48	48	48	Slightly mottled and irridescent		Negative
Modified haemin		26	35	48	50	50	50	Small to large non-mucoid greenish brown		Positive after 4 days
Sheffield	2	40	47	50	50	50	50	Large non-mucoid greenish brown	Rail road tracks common	Positive after 2 days

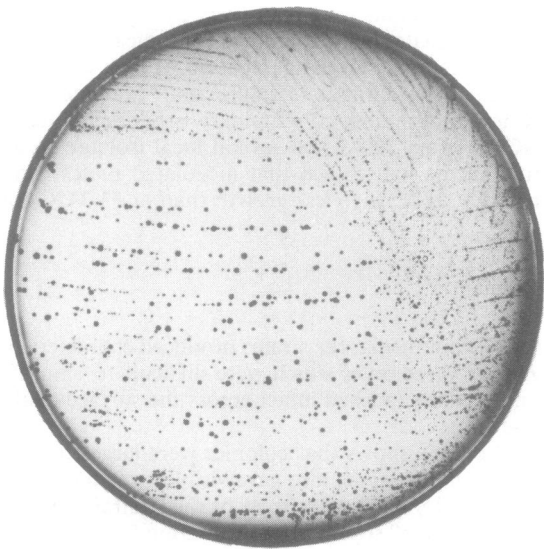

FIGURE *Typical rail road tracks appearance of H ducreyi colony growing on new Sheffield medium.*

lower incubation temperature of 33-34°C was necessary for optimum growth of *H ducreyi.*

In this study the improved Sheffield medium proved superior to the others tested in terms of time to first visible growth, while maintaining the characteristic colonial morphology of *H ducreyi.* As well as giving larger colonial size, it enabled starch aggregation to be used as an additional means of identifying an otherwise inert organism. The starch aggregation reaction, which facilitates recognition of even single colonies of *H ducreyi* in heavily contaminated plates, is negative in all media containing serum, possibly because of starch binding.[6] The addition of vancomycin (3 mg/l) to the medium inhibits the growth of Gram positive organisms from the polymicrobial flora of genital ulcers, and is advocated for the primary isolation of *H ducreyi* from clinical specimens. Use of this medium in STD clinics may increase the number of confirmed diagnoses of chancroid.

References

1. McEntegart MG, Hafiz S, Kinghorn GR. *Haemophilus ducreyi* infections—time for reappraisal. *Journal of Hygiene (Cambridge),* 1982;**89**:467-78.
2. Hammond GW, Lian CJ, Wilt JC, Ronald AR. Comparison of specimen collection and laboratory techniques for isolation of *Haemophilus ducreyi. J Clin Microbiol* 1978;**7**:39-43.
3. Sottnek FO, Biddle JW, Kraus SJ, Weaver RE, Stewart JA. Isolation and identification of *Haemophilus ducreyi* in a clinical study. *J Clin. Microbiol* 1980;**12**:170-4.
4. Hafiz S, Kinghorn GR, McEntegart MG. Chancroid in Sheffield. *Br J Vener Dis* 1981;**57**:382-6.
5. Kinghorn GR, Hafiz S, McEntegart MG. Modified haemin containing medium for isolation of *Haemophilus ducreyi. Lancet* 1982;i:383-4.
6. Hafiz S, Kinghorn GR, McEntegart MG. Starch aggregation as a presumptive test for *Haemophilus ducreyi. Lancet* 1982;ii:872.